PROCESS FOR THE HYDROLYSIS OF STARCH

FIELD OF THE INVENTION

The present invention relates to a process for hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

BACKGROUND OF THE INVENTION

A large number of processes have been described for converting starch to starch hydrolysates, such as maltose, glucose or specialty syrups, either for use as sweeteners or as precursors for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products, such as citric acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta lactone, sodium erythorbate, itaconic acid, lactic acid, gluconic acid; ketones; amino acids, glutamic acid (sodium monoglutaminate), penicillin, tetracyclin; enzymes; vitamins, such as riboflavin, B12, beta-carotene or hormones.

Starch is a high molecular-weight polymer consisting of chains of glucose units. It usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains of alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. In the case of converting starch into a soluble starch hydrolysate, the starch is depolymerized. The conventional depolymerization process consists of a gelatinization step and two consecutive process steps, namely a liquefaction process and a saccharification process.

Granular starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation. During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase. The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The temperature is then lowered to 60°C, a glucoamylase or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase are added, and the saccharification process proceeds for about 24 to 72 hours.

It will be apparent from the above discussion that the conventional starch conversion process is very energy consuming due to the different requirements in terms of

temperature during the various steps. It is thus desirable to be able to select the enzymes used in the process so that the overall process can be performed without having to gelatinize the starch. Such processes are the subject for the patents US4591560, US4727026 and US4009074 and EP0171218.

The present invention relates to a one-step process for converting granular starch into soluble starch hydrolysate at a temperature below initial gelatinization temperature of the starch.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the action of a first enzyme, which enzyme; is a member of the Glycoside Hydrolase Family 13; has alpha-1.4-glucosidic hydrolysis activity, and; comprises a functional Carbohydrate-Binding Module (CBM) belonging to CBM Family 20, which CBM has an amino acid sequence having at least 60% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3; and which second enzyme is selected from the list comprising a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), and a glucoamylase (E.C.3.2.1.3).

The process of the first aspect of the invention may be performed as a one step process and/or as a process comprising one or more steps.

In a second aspect the invention provides a process for production of high fructose starch-based syrup (HFSS), the process comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for conversion of the soluble starch hydrolysate into a high fructose starch-based syrup (HFSS).

In a third aspect the invention provides a process for production of fuel or potable ethanol; comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for fermentation of the soluble starch hydrolysate into ethanol, wherein the fermentation step is carried out simultaneously or separately/ sequential to the hydrolysis of the granular starch.

In a fourth aspect the invention provides a use of an enzyme having alpha-amylase activity in a process for hydrolysis of starch, said enzyme comprising a functional CBM having an amino acid sequence having at least 60% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.

In a firth aspect the invention provides a use of an enzyme having alpha-amylase activity in a process for hydrolysis of granular starch, said enzyme comprising an amino acid sequence having at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%, such as at least 99% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ

ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18.

In a sixth aspect the invention provides a use of an enzyme having alpha-amylase activity and a functional CBM in a process for hydrolysis of granular starch, said enzyme comprising an amino acid sequence having at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%, such as at least 99% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO:22.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "granular starch" is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization. Starch is formed in plants as tiny granules insoluble in water. These granules are preserved in starches at temperatures below the initial gelatinization temperature. When put in cold water, the grains may absorb a small amount of the liquid. Up to 50°C to 70°C the swelling is reversible, the degree of reversibility being dependent upon the particular starch. With higher temperatures an irreversible swelling called gelatinization begins.

The term "initial gelatinization temperature" is understood as the lowest temperature at which gelatinization of the starch commences. Starch heated in water begins to gelatinize between 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch and can readily be determined by the skilled artisan. Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch is the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein. S. and Lii. C., Starch/Stärke, Vol. 44 (12) pp. 461-466 (1992).

The term "soluble starch hydrolysate" is understood as the soluble products of the processes of the invention and may comprise mono- di-, and oligosaccharides, such as glucose, maltose, maltodextrins, cyclodextrins and any mixture of these. Preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

The term "Speciality Syrups", is an in the art recognised term and is characterised according to DE and carbohydrate spectrum (See the article "New Speciality Glucose Syrups", p. 50+, in the textbook "Molecular Structure and Function of Food Carbohydrate", Edited by G.G. Birch and L.F. Green, Applied Science Publishers LTD., London). Typically Speciality Syrups have a DE in the range from 35 to 45.

The "Glycoside Hydrolase Family 13" is in the context of this invention defined as the group of hydrolases comprising a catalytic module having a (beta/alpha)8 or TIM barrel structure and acting on starch and related substrates through an alpha-retaining reacting mechanism (Koshland, 1953, Biol.Rev.Camp.Philos.Soc 28, 416-436).

The enzymes having "alpha-1.4-glucosidic hydrolysis activity" is in the context of this invention defined as comprising the group of enzymes which catalyze the hydrolysis and/or synthesis of alpha-1,4-glucosidic bonds as defined by Takata (Takata et al, 1992, J. Biol. Chem. 267, 18447-18452) and by Koshland (Koshland, 1953, Biol.Rev. Camp. Philos. Soc 28, 416-436).

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683. The numbering of Glycoside Hydrolase Families applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *"Genetics, Biochemistry and Ecology of Cellulose Degradation"*., K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23, and Bourne, Y. & Henrissat, B. 2001; Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600.

A carbohydrate-binding module (CBM) is a polypeptide amino acid sequence which binds preferentially to a poly- or oligosaccharide (carbohydrate), frequently - but not necessarily exclusively - to a water-insoluble (including crystalline) form thereof.

Although a number of types of CBMs have been described in the patent and scientific literature, the majority thereof - many of which derive from cellulolytic enzymes (cellulases) - are commonly referred to as "cellulose-binding modules"; a typical cellulose-binding module will thus be a CBM which occurs in a cellulase. Likewise, other sub-classes of CBMs would embrace, e.g., chitin-binding modules (CBMs which typically occur in chitinases), xylan-binding modules (CBMs which typically occur in xylanases), mannan-binding modules (CBMs which typically occur in mannanases), starch-binding modules (CBMs which may occur in certain amylolytic enzymes, such as certain glucoamylases, or in enzymes such as cyclodextrin glucanotransferases), or in alpha-amylases.

CBMs are found as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic module containing the active site for substrate hydrolysis and a carbohydrate-binding module (CBM) for binding to the

carbohydrate substrate in question. Such enzymes can comprise more than one catalytic module and one, two or three CBMs, and optionally further comprise one or more polypeptide amino acid sequence regions linking the CBM(s) with the catalytic module(s), a region of the latter type usually being denoted a "linker". Examples of hydrolytic enzymes comprising a CBM - some of which have already been mentioned above - are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. CBMs have also been found in algae, e.g. in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein.

In proteins/polypeptides in which CBMs occur (e.g. enzymes, typically hydrolytic enzymes), a CBM may be located at the N or C terminus or at an internal position.

That part of a polypeptide or protein (e.g. hydrolytic enzyme) which constitutes a CBM *per se* typically consists of more than about 30 and less than about 250 amino acid residues.

Preferred for the invention are enzymes comprising a CBM comprising an amino acid sequence selected from the group consisting of amino acid sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 as well as enzymes comprising a CBM comprising an amino acid sequence having at least 50% homology to an amino acid sequence selected from the group consisting of amino acid sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3

The polypeptide "homology" referred to in this disclosure is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for amino acid sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

The enzyme to be used as a first enzyme of the present invention is a four module alpha-amylase consisting of a three module amylase core and a separate carbohydrate binding module of family 20. The alpha-amylase may be a wild type alpha-amylase derived from bacterial or fungal sources, or it may be mutants, protein engineered variants, or other variants of such wild types, or it may be hybrids of variants or wild types.

Preferably the alpha-amylase is a wild type enzyme. More preferably the alpha-amylase is a variant and/or hybrid of the above alpha-amylases comprising amino acid modifications leading to increased activity, increased protein stability at low pH, and/or at high pH, increased stability towards calcium depletion, and/or increased stability at elevated temperature.

The term "Enzyme hybrids" referred to in this disclosure is understood as modified enzymes comprising an amino acid sequence of an amylolytic enzyme [which in the context of the present invention may, e.g., be an alpha-amylase (EC 3.2.1.1), an isoamylase (EC 3.2.1.68) or a pullulanase (EC 3.2.1.41)] linked (i.e. covalently bound) to an amino acid

sequence comprising a CBM. The CBM is preferably but not exclusively fused to the N-terminal. The hybrid may comprise more than one CBM.

CBM-containing enzyme hybrids, as well as detailed descriptions of the preparation and purification thereof, are known in the art [see, e.g., WO 90/00609, WO 94/24158 and WO 95/16782, as well as Greenwood et al., Biotechnology and Bioengineering 44 (1994) pp. 1295-1305]. They may, e.g., be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding module ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest, and growing the transformed host cell to express the fused gene.

The construction of a hybrid protein between a carbohydrate binding module (CBM) and an alpha-amylase requires one or more of the following steps to obtain a stable, expressible and applicable enzyme.

1) Aligning the CBM-donor molecule with the donor of the catalytic modules using conventional methods is often required to identify possible crossing points. If the homology is relatively high there might be several possible crossing point. If however the homology is low or if only the sequence of the catalytic module and the CBM are available, respectively, the CBM can be attached as an elongation to the catalytic module either in the beginning of the sequence, i.e. in the N-terminal inserted after an eventually signal sequence; or in the C-terminal prior to the termination signal. Regardless if the CBM is located in the N- or in the C-terminal it might be beneficial to either delete a few amino acids or insert a number of amino acid as a linker to obtain an expressible and application stable enzyme.

2) Construction the DNA hybrid of the genes coding for the CBM and the amylolytic module according to the considerations made under 1) can be made by methods known to persons skilled in the art. These methods include among others, PCR reactions using primers designed to hybridize over the resulting DNA crossing point, DNA digesting followed by ligation or in-vivo combination for example by yeast.

3) A simple attachment of a CBM to an amylolytic module often results in a hybrid protein that is expressed poorly due to folding or stability problems or in a hybrid protein lacking sufficient stability and/or activity under a given application. To overcome such problems the hybrid protein may be subjected to protein engineering either by site directed mutagenesis methods or by more random approaches. This includes both the amino acids in the modules of the CBM and in the amylolytic modules as well as optimizing the transition from amylolytic module to CBM, with respect to length and amino acid sequence.

Preferred as a first enzyme for the present invention are hybrid enzymes comprising a CBM comprising an amino acid sequence selected from the group consisting of amino acid sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 as well as enzymes comprising an amino acid sequence having at least 50% at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%,

such as at least 99% homology to an amino acid sequence selected from the group consisting of the amino acid sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.

Also preferred as a first enzyme for the present invention are hybrid enzymes comprising an amino acid sequence having alpha-amylolytic activity and comprising an amino acid sequence selected from the group consisting of amino acid sequences SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18 as well as enzymes comprising an amino acid sequence having at least 70%, at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%, such as at least 99% homology to an amino acid sequence selected from the group consisting of amino acid sequences SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18.

Preferably the first enzyme of the present invention comprises a CBM and/or an alpha-amylolytic sequence derived from a fungi, such as from a strain belonging to a *Talaromyces* sp., or from a strain belonging to an *Aspergillus* sp. such as *A.awamori, A. kawachii, A.niger, A.oryzae* etc. or from a bacteria, such as from a strain belonging to *Bacillus* sp, such as from a strain belonging to *B. amyloliquefacience*, *B. flavothermus*, *B. licheniformis* or *B. stearothermophilus*,

More preferred as a first enzyme of the present invention is a four module alpha-amylase consisting of a three module amylase core and a separate carbohydrate binding module of family 20. Most preferred is a four module alpha-amylase comprising an amino acid sequence having at least 70%, at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%, such as at least 99% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO:22.

Preferably the first enzyme of the present invention is a four module alpha-amylase isolated from a fungus or a bacteria, such as from a species of *Bacillus* sp, such as the polypeptides shown in SEQ ID NO:20, and SEQ ID NO:21, or from a strain of *Bacillus flavothermus*, such as the polypeptide shown in SEQ ID NO:19, or from a strain of *Aspergillus kawachii* such as the polypeptide shown in SEQ ID NO:22.

Most preferred as a first of the present invention is an alpha-amylase comprising an amino acid sequence having at least 70%, at least 75%, least 80%, at least 85%, at least 90%, least 95%, at least 98%, such as at least 99% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO:22.

The above alpha-amylases may be added in an amount of 0.001-1.0 KNU/g DS, preferably from 0.002-0.5 KNU/g DS, preferably 0.02-0.1 KNU/g DS.

Fungal alpha-amylase

A particular enzyme to be used as a second enzyme in the processes of the invention is a fungal alpha-amylase (EC 3.2.1.1), such as a fungamyl-like alpha-amylase. In the present disclosure, the term "fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high homology, i.e. more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID No. 10 in WO96/23874. Fungal alpha-amylases may be added in an amount of 0.001-1.0 AFAU/g DS, preferably from 0.002-0.5 AFAU/g DS, preferably 0.02-0.1 AFAU/g DS.

Beta-amylase

Another particular enzyme to be used as a second enzyme in the processes of the invention may be a beta-amylase (E.C 3.2.1.2). Beta-amylase is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers thereby releasing maltose.

Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7.0. Contemplated beta-amylase include the beta-amylase from barley Spezyme® BBA 1500, Spezyme® DBA and Optimalt™ ME, Optimalt™ BBA from Genencor Int. as well as Novozym™ WBA from Novozymes A/S.

Glucoamylase

A further particular enzyme to be used as a second enzyme in the processes of the invention may also be a glucoamylase (E.C.3.2.1.3) derived from a microorganism or a plant. Preferred is glucoamylases of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO92/00381 and WO00/04136; the *A. awamori* glucoamylase (WO84/02921), *A. oryzae* (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), *Prot. Engng.* 9, 499-505); D257E and D293E/Q (Chen et al. (1995), *Prot. Engng.* 8, 575-582); N182 (Chen et al. (1994), *Biochem. J.* 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein *Engng.* 10, 1199-1204. Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO99/28448), *Talaromyces leycettanus* (US patent no. Re.32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215). Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C.*

thermoamylolyticum (EP135,138), and *C. thermohydrosulfuricum* (WO86/01831). Preferred glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136. Also contemplated are the commercial products AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (from Novozymes); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900 (from Enzyme Bio-Systems); G-ZYME™ G990 ZR (*A. niger* glucoamylase and low protease content).

Glucoamylases may be added in an amount of 0.02-2.0 AGU/g DS, preferably 0.1-1.0 AGU/g DS, such as 0.2 AGU/g DS.

Additional enzymes.

The processes of the invention may be carried out in the presence of a third enzyme. A particular third enzyme may be a *Bacillus* alpha-amylase (often referred to as "Termamyl-like alpha-amylases"). Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available as Termamyl), *B. amyloliquefaciens,* and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO96/23874, WO97/41213, and WO99/19467. Specifically contemplated is a recombinant *B. stearothermophilus* alpha-amylase variant with the mutations: I181* + G182* + N193F. *Bacillus* alpha-amylases may be added in effective amounts well known to the person skilled in the art.

Another particular third enzyme of the process may be a debranching enzyme, such as an isoamylase (E.C. 3.2.1.68) or a pullulanases (E.C. 3.2.1.41). Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by the limited action on alpha-limit dextrins. Debranching enzyme may be added in effective amounts well known to the person skilled in the art.

Embodiments of the invention

The starch slurry to be subjected to the processes of the invention may have 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids granular starch.

After being subjected to the process of the first aspect of the invention at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least

92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or preferably at least 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

According to the invention the processes of the first and second aspect is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which the processes are conducted is at least 30°C, at least 31°C, at least 32°C, at least 33°C, at least 34°C, at least 35°C, at least 36°C, at least 37°C, at least 38°C, at least 39°C, at least 40°C, at least 41°C, at least 42°C, at least 43°C, at least 44°C, at least 45°C, at least 46°C, at least 47°C, at least 48°C, at least 49°C, at least 50°C, at least 51°C, at least 52°C, at least 53°C, at least 54°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, or preferably at least 60°C.

The pH at which the process of the first aspect of the invention is conducted may in be in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

The exact composition of the products of the process of the first aspect of the invention, the soluble starch hydrolysate, depends on the combination of enzymes applied as well as the type of granular starch processed. Preferably the soluble hydrolysate is maltose with a purity of at least 85%, at least 90%, at least 95.0%, at least 95.5%, at least 96.0%, at least 96.5%, at least 97.0%, at least 97.5%, at least 98.0%, at least 98.5, at least 99.0% or at least 99.5%. Even more preferably the soluble starch hydrolysate is glucose, and most preferably the starch hydrolysate has a DX (glucose percent of total solubilised dry solids) of at least 94.5%, at least 95.0%, at least 95.5%, at least 96.0%, at least 96.5%, at least 97.0%, at least 97.5%, at least 98.0%, at least 98.5, at least 99.0% or at least 99.5%. Equally contemplated, however, is the process wherein the product of the process of the invention, the soluble starch hydrolysate, is a speciality syrup, such as a speciality syrup containing a mixture of glucose, maltose, DP3 and DPn for use in the manufacture of ice creams, cakes, candies, canned fruit.

The granular starch to be processed in the processes of the invention may in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically the granular starch may be obtained from corns, corn grits, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley. The granular starch to be processed may be a highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5 % pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibres. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where the starch hydrolysate is used in production of syrups.

Both dry and wet milling is well known in the art of starch processing and are equally contemplated for the processes of the invention. The process of the first aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Also contemplated is the process conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

In the process of the second aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS). This conversion is preferably achieved using a glucose isomerase, and more preferably by an immobilized glucose isomerase supported on a solid support. Contemplated isomerases comprises the commercial products Sweetzyme™ IT from Novozymes A/S, G -zyme™ IMGI and G-zyme™ G993, Ketomax™ and G-zyme™ G993 from Rhodia, G-zyme™ G993 liquid and GenSweet™ IGI from Genencor Int.

In the process of the third aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is used for production of fuel or potable ethanol. In the process of the third aspect the fermentation may be carried out simultaneously or separately/sequential to the hydrolysis of the granular starch slurry. When the fermentation is performed simultaneous to the hydrolysis the temperature is preferably between 30°C and 35°C, and more preferably between 31°C and 34°C. The process of the third aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

The soluble starch hydrolysate of the process of the first aspect of the invention may also be used for production of a fermentation product comprising fermenting the treated starch into a fermentation product, such as citric acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta lactone, or sodium erythorbate.

In another embodiment the starch slurry is being contacted with a polypeptide comprising a CBM, but no amylolytic module, i.e. application of loose CBMs. The loose

CBMs may be starch binding modules, cellulose-binding modules, chitin-binding modules, xylan-binding modules, mannan-binding modules, and other binding modules. Preferred CBMs in the present context are microbial CBMs, particularly bacterial or fungal CBMs. Particularly preferred are the starch binding modules shown in the present disclosure as the polypeptide sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 or the starch binding modules disclosed in US provisional application No. 60/511044 as SEQ ID NO:12; the CBM of the glucoamylase from *Hormoconis* sp. such as from *Hormoconis resinae* (Syn. Creosote fungus or *Amorphotheca resinae*) (SWISSPROT:Q03045), SEQ ID NO:13; the CBM from *Lentinula* sp. such as from *Lentinula edodes* (shiitake mushroom) (SPTREMBL:Q9P4C5), SEQ ID NO:14; the CBM from *Neurospora* sp. such as from *Neurospora crassa* (SWISSPROT:P14804), SEQ ID NO:15; the CBM from *Talaromyces* sp. such as from *Talaromyces byssochlamydioides*, SEQ ID NO:16; the CBM from *Geosmithia* sp. such as from *Geosmithia cylindrospora*, SEQ ID NO:17: the CBM from *Scorias* sp. such as from *Scorias spongiosa*, SEQ ID NO:18; the CBM from *Eupenicillium* sp. such as from *Eupenicillium ludwigii*, SEQ ID NO:19; the CBM from *Aspergillus* sp. such as from *Aspergillus japonicus*, SEQ ID NO:20; the CBM from *Penicillium* sp. such as from *Penicillium* cf. *miczynskii*, SEQ ID NO:21; the CBM from Mz1 *Penicillium* sp., SEQ ID NO:22; the CBM from *Thysanophora* sp., SEQ ID NO:23; the CBM from *Humicola* sp. such as from *Humicola grisea var. thermoidea*. Most preferred CBMs include the CBMs disclosed in US provisional application No. 60/511044 as SEQ ID NO:24; the CBM of the glucoamylase from *Aspergillus* sp. such as from *Aspergillus niger*, and as SEQ ID NO:25; the CBM of the glucoamylase from *Athelia* sp. such as from *Athelia rolfsii*. Also preferred for the invention is the application of any CBM having at least 50%, 60%, 70%, 80% or even at least 90% homology to any of the afore mentioned CBM amino acid sequences.

The loose CBMs may be applied to the granular starch slurry in effective amounts.

MATERIALS AND METHODS

Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue colour is formed, but during the break-down of the starch the blue colour gets weaker and gradually turns into a reddish-brown, which is compared to a coloured glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5.26 g starch dry substance Merck Amylum solubile.

A folder AF 9/6 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A folder (AEL-SM-0131) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Fungal alpha-amylase activity (FAU)

Fungal alpha-amylase activity may be measured in FAU (Fungal Alpha-Amylase Units). One (1) FAU is the amount of enzyme which under standard conditions (i.e. at 37°C and pH 4.7) breaks down 5260 mg solid starch (Amylum solubile, Merck) per hour. A folder AF 9.1/3, describing this FAU assay in more details, is available upon request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (from Novozymes A/S, glucoamylase wildtype Aspergillus niger G1, also disclosed in Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102 and in WO92/00381). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with the following description. In this method 1 AFAU is defined as the amount of enzyme, which degrades 5.26 mg starch dry solids per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

Starch + Iodine	Alpha-amylase ⟶ 40°C, pH 2.5	Dextrins + Oligosaccharides
Blue/violet	t=23 sec.	Decoloration

Standard conditions/reaction conditions: (per minute)

Substrate:	starch, approx. 0.17 g/L
Buffer:	Citate, approx. 0.03 M
Iodine (I2):	0.03 g/L
CaCl2:	1.85 mM
pH:	2.50 - 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	lambda=590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

If further details are preferred these can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, and incorporated by reference.

Beta-amylase activity (DP°)

The activity of SPEZYME® BBA 1500 is expressed in Degree of Diastatic Power (DP°). It is the amount of enzyme contained in 0.1 ml of a 5% solution of the sample enzyme preparation that will produce sufficient reducing sugars to reduce 5 ml of Fehling's solution when the sample is incubated with 100 ml of substrate for 1 hour at 20°C.

Pullulanase activity (New Pullulanase Unit Novo (NPUN)

Pullulanase activity may be determined relative to a pullulan substrate. Pullulan is a linear D-glucose polymer consisting essentially of maltotriosyl units joined by 1,6-alpha-links. Endo-pullulanases hydrolyze the 1,6-alpha-links at random, releasing maltotriose, 6^3-alpha-maltotriosyl-maltotriose, 6^3-alpha-(6^3-alpha-maltotriosyl-maltotriosyl)-maltotriose.

One new Pullulanase Unit Novo (NPUN) is a unit of endo-pullulanase activity and is measured relative to a Novozymes A/S Promozyme D standard. Standard conditions are 30 minutes reaction time at 40°C and pH 4.5; and with 0.7% pullulan as substrate. The amount of red substrate degradation product is measured spectrophotometrically at 510 nm and is proportional to the endo-pullulanase activity in the sample. A folder (EB-SM.0420.02/01)

describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Under the standard conditions one NPUN is approximately equal to the amount of enzyme which liberates reducing carbohydrate with a reducing power equivalent to 2.86 micromole glucose per minute.

Determination of sugar profile and solubilised dry solids

The sugar composition of the starch hydrolysates was determined by HPLC and glucose yield was subsequently calculated as DX. °BRIX, solubilised (soluble) dry solids of the starch hydrolysate were determined by refractive index measurement.

Materials

The following enzyme activities were used. A bacterial alpha-amylase with a CBD having the sequence depicted in SEQ ID NO:19 and the same bacterial alpha-amylase but without the CBD module (SEQ ID NO:4). A glucoamylase derived from *Aspergillus niger* having the amino acid sequence shown in WO00/04136 as SEQ ID No: 2 or one of the disclosed variants. An acid fungal alpha-amylase derived from *Aspergillus niger.*

Wheat starch (S-5127) was obtained from Sigma-Aldrich.

Example 1

This example illustrates the conversion of granular wheat starch into glucose using a bacterial four module alpha-amylase and a glucoamylase and an acid fungal amylase. A slurry with 33% dry solids (DS) granular starch was prepared by adding 247.5 g of wheat starch under stirring to 502.5 ml of water. The pH was adjusted with HCl to 4.5. The granular starch slurry was distributed to 100 ml blue cap flasks with 75 g in each flask. The flasks were incubated with magnetic stirring in a 60°C water bath. At zero hours the enzyme activities given in table 1 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours.

Table 1. The enzyme activity levels used.

Bacterial alpha-amylase KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
100.0	200	50

Total dry solids starch was determined using the following method. The starch was completely hydrolyzed by adding an excess amount of alpha-amylase (300 KNU/Kg dry

solids) and placing the sample in an oil bath at 95 °C for 45 minutes. Subsequently the samples were cooled to 60°C and an excess amount of glucoamylase (600 AGU/kg DS) was added followed by incubation for 2 hours at 60°C.

Soluble dry solids in the starch hydrolysate were determined by refractive index measurement on samples after filtering through a 0.22 microM filter. The sugar profile were determined by HPLC. The amount of glucose was calculated as DX. The results are shown in table 2 and 3:

Table 2. Soluble dry solids as percentage of total dry substance at 100 KNU/kg DS alpha-amylase dosage.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
100.0	92.5	96	97.3	99.2

Table 3. The DX of the soluble hydrolysate at 100 KNU/kg DS alpha-amylase dosage.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
100.0	88.4	92.4	93.7	95.3

Example 2

This example illustrates the only partial conversion of granular starch into glucose using a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 4 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours. The samples were analyzed as described in examples 1. The results are shown in table 5 and 6.

Table 4. The enzyme activity level used.

Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
200	50

Table 5. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
28.5	36.3	41.6	45.7

Table 6. DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
27.7	34.9	39.2	42.2

Example 3

In example 3 conversion of granular wheat starch into glucose was performed using a glucoamylase (200 AGU/kg DS), an acid fungal amylase (50 AFAU/kg DS) and either the intact bacterial four module alpha-amylase (SEQ ID NO:19) also used in example 1 or the same bacterial four module alpha-amylase but without the CBD module (SEQ ID NO:4) (100 KNU/kg DS). A slurry with 33% dry solids (DS) granular starch was prepared and incubated as described in example 1. Samples were withdrawn after 24, 46, 70, and 90 hours.

Total dry solids starch was determined as described in example 1. Soluble dry solids in the starch hydrolysate and the sugar profile were determined as described in example 1. The results are shown in table 7 and 8.

Table 7. Soluble dry solids as percentage of total dry substance. Enzymes: glucoamylase, fungal acid amylase and bacterial alpha-amylase with the CBD module (SEQ ID NO:19) or without the CBD module (SEQ ID NO:4).

	24 hours	46 hours	70 hours	90 hours
Without CBD	89,7	92,4	92,4	92,5
With CBD	94,1	95,2	96,9	97,1

Table 8. The DX of the soluble hydrolysate: Enzymes: glucoamylase, fungal acid amylase and bacterial alpha-amylase with the CBD module (SEQ ID NO:19) or without the CBD module (SEQ ID NO:4).

	24 hours	46 hours	70 hours	90 hours
Without CBD	85,9	88,7	89,0	89,0
With CBD	89,9	93,3	93,0	93,2